Definition of a Natural Killer NKR-P1A$^+$/CD56$^-$/CD16$^-$ Functionally Immature Human NK Cell Subset That Differentiates In Vitro in the Presence of Interleukin 12

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Summary

Human natural killer (NK) cell differentiation from immature lineage negative (Lin$^-$) umbilical cord blood cells was examined in vitro. Cells expressing differentiation antigens of mature NK cells (CD56, CD16, CD2, CD8, NKR-P1A) were generated from Lin$^-$ cells cultured with interleukin (IL)-2 and a murine bone marrow stromal cell line expressing the human membrane-bound form of stem cell factor. Two subsets of NK cells were identified in these cultures: one expressed both NKR-P1A and CD56 and, in variable proportions, all other NK cell differentiation antigens; the second subset expressed only NKR-P1A and, unlike the former, was not cytotoxic. Neither subset expressed interferon (IFN)-γ mRNA even after stimulation with phorbol di-ester and Ca^{2-} ionophore, but both expressed tumor necrosis factor α mRNA and the cytotoxic granule-associated proteins TIA-1, perforin, and serine esterase-1. After 10-d culture with IL-2, IL-12, and irradiated B lymphoblastoid cells, ≈45% of the NKR-P1A$^+$/CD56$^-$ cells became CD56$^+$, and the same cultures contained cells capable of cytotoxicity and of IFN-γ production. These results indicate that NKR-P1A expression in the absence of other NK cell markers defines an intermediate, functionally immature stage of NK cell differentiation, and that effector functions develop in these cells, concomitantly with CD56 expression, in the presence of IL-12. These cells likely represent the counterpart of a CD3$^-$/NKR-P1A$^+$/CD56$^-$/CD16$^-$ cell subset that, as shown here, is present both in adult and neonatal circulating lymphocytes.

NK cells are a lymphocyte subset distinct from B and T cells, lacking expression of the antigen-specific receptors present on these cell types (T and B cell receptors, i.e., TCR and sIg/BCR) (reviewed in reference 1). They represent ≈15% of the circulating lymphocytes in adult and neonatal peripheral blood (2), are effectors of nonadaptive immunity, are cytotoxic, in the absence of known presensitization, to a variety of target cells, and, via IFN-γ production, they facilitate the development of Th0 to Th1 cells (3), thus promoting cell-mediated immune responses (4). No unique NK cell marker has been identified yet, but expression of a set of differentiation antigens in the absence of antigen-specific receptors of T and B lymphocytes serves to identify these cells. CD16 and CD56 are expressed, either alone or in combination, on the majority of mature NK cells (2). Other antigens expressed on subsets of NK cells include CD8 (30–50%) (5), and CD2 (50–70% of NK cells from umbilical cord blood and >90% of NK cells from adult blood) (2).

Human NK cell progenitors exist in the lineage negative (Lin$^-$)[1]/CD34$^+$ subset of hematopoietic cells that includes multilineage progenitors and hematopoietic stem cells (6–10). A variety of in vitro culture conditions, requiring IL-2, IL-15, and/or bone marrow stromal cells (7, 8) or stem cell factor (SCF) (9, 10), or IL-15 (11) support differentiation of these cells to mature CD56$^+$ cytotoxic NK cells. CD7 is expressed on early NK cell progenitors; these have growth factor requirements distinct from those of more differentiated cells: CD7$^+$/CD34$^+$ NK cell progenitors require bone marrow stromal cells and IL-2 to differentiate into CD56$^+$ cytotoxic cells (7), while CD7$^+$/CD34$^-$/CD56$^-$ cells, representing more mature progenitor cells in umbilical cord blood, require only IL-2 (12). In the murine system, NK cells at distinct stages of differentiation have been identified based on surface phenotype; non–stem cell hematopoietic

[1] *Abbreviations used in this paper*: ADCC, antibody-dependent cell-mediated cell toxicity; GaMIg, goat anti–mouse Ig; Lin$^-$, lineage negative; MSE, monocyte-specific esterase; RT, reverse transcription, SCF, stem cell factor; SE, serine esterase

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progenitors lacking expression of mature NK cell surface antigens develop into mature cytotoxic NK cells in adoptive transfer experiments (13, 14). Conjugate-forming, noncytotoxic, putative pre-NK cells that express NKR-P1C (NK1.1) in the absence of other mature NK cell markers have been identified in mice in which, after bone marrow ablation, NK cell differentiation is incomplete (15).

Differentiation antigens that identify human NK cells at intermediate stages of differentiation at which they have unequivocal, although possibly incomplete, NK cell phenotype, and at which they are functionally immature have not been described. In addition, the specific sequence with which human NK cells acquire differentiation antigens and/or functions, and the growth factors supporting NK cell development/differentiation have been determined only in part. Here we report the identification of a functionally immature, noncytotoxic NK cell subset, characterized by the expression of NKR-P1A and lack of other NK cell differentiation antigens, that can be induced to differentiate to functionally and phenotypically mature NK cells in the presence of IL-12. A subset of adult and neonatal circulating NK cells was also identified, that has a surface phenotype similar, if not identical, to that of the immature NK cells generated in vitro.

Materials and Methods

Monoclonal and Polyclonal Antibodies. Most mAbs used have been characterized previously in our laboratory. They include: CD2 (B67.1, and B67.6), CD4 (B66.6), CD5 (B36.1), CD8 (B116.1), CD11b (B43.4), CD14 (B52.1), CD15 (B40.9), and CD56 (B159.5) (2, 16). mAbs to CD3 (OKT3) (17), CD21 (THB5) (18), and CD34 (MY10) (19) were produced from cells obtained from American Type Culture Collection (Rockville, MD). mAb to CD94 (HP3B1) (20) was kindly provided by Dr. M. Lopez-Botet (Hospital de la Princesa, Madrid, Spain). PE-anti-CD16 mAb (B73.1-PE) (2) was from Becton Dickinson and Co. (San Jose, CA). PE-anti-CD56 (N901) and the FITC-labeled mAb to CD7 (3A1), CD8 (T8), CD25 (1HT44H3), and CD122 (2RB) were from Coulter Corp. (Hialeah, FL).

mAb B199.2 was produced from a fusion, performed as previously reported (2), of the P3x63-Ag8.653 nonsecretor myeloma cells with splenocytes from a BALB/c mouse immunized (three times intraperitoneally at weekly intervals, and once intravenously 3 d before fusion) with purified human NK cells, prepared as described previously (16) and cultured for 3 d with IL-2. This mAb (IgG2b, as determined by immunodiffusion with isotype-specific antibodies) detects NKR-P1A on NK cells and subsets of $CD4^+$ and $CD8^+$ T cells (not shown), as indicated by the observations that: (*a*) it immunoprecipitates from mature NK cells an 80-kD surface molecule, a disulfide-linked homodimer of two 40-kD chains (Zatsepina, O., and B. Perussia, unpublished data); (*b*) it cross-competes with the anti-NKR-P1A mAb DX-1 (21) for binding to NK cells; and (*c*) it reacts with NKR-P1A–transfected, but not untransfected, murine L cells (assay kindly performed by Dr. L. Lanier, DNAX, Palo Alto, CA). When indicated, this and other mAbs were labeled with biotin or FITC according to standard procedures, after purification from ascites on protein G–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) columns.

The polyclonal goat anti–mouse Ig (GaMIg) used for panning was produced in our laboratory, adsorbed on human Ig and affinity-purified on mouse Ig-Sepharose before use (16). The rabbit IgG anti–sheep erythrocyte used to prepare immune complex monolayers was from Cappel (Durham, NC).

Cell Isolation. Umbilical cord blood, collected at delivery and anticoagulated with heparin, was kindly provided by Dr. R. Depp (Department of Obstetrics and Gynecology, Thomas Jefferson University Hospital, Philadelphia, PA). PBMC were isolated on Ficoll/Hypaque density gradient (Histopaque-1077; Sigma Chemical Co., St. Louis, MO) and contaminating erythrocytes were lysed using NH_4Cl buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM EDTA; 4°C, 10 min). PBL were isolated from PBMC after adherence to plastic (37°C, 20 min). The majority of T and NK cells were depleted after rosetting with 2-aminoethylisothiouronium bromide (hydrdoromide) (Sigma Chemical Co.)-treated sheep E (21). PBL not forming E-rosettes were collected at the interface of Ficoll/Hypaque density gradients and $Fc\gamma R^+$ (NK, B, and myelo-monocytic) cells were depleted after incubation on rabbit IgG immune complex monolayers as described (22). Residual mature hematopoietic cells were depleted from the $Fc\gamma R^-$ cells by indirect anti-Ig panning (23) (30 min, 4°C) on GaMIg-treated polystyrene petri dishes after sensitization with a panel of mAbs to differentiation antigens on mature hematopoietic cells, including mAb (clones listed above) to CD2, CD3, CD4, CD5, CD8, CD11b, CD14, CD15, CD21, CD56, CD94, and NKR-P1A. The procedure was repeated three times to maximize depletion. These populations, referred to as Lin^- cells, were >99% $CD3^-/CD56^-/NKR\text{-}P1A^-/CD16^-$; the majority of them (81.2 ± 9.7%, $n = 9$) were HLA-DR^+ and contained 57.6 ± 19.7% ($n = 7$) $CD34^+$ cells, as determined by indirect immunofluorescence.

When indicated, resting NK cells were prepared from adult and umbilical cord blood PBL after sensitization with a mixture of mAb to CD3, CD4, CD5, CD14, CD15, CD21, and CD34 and panning. The resulting cell populations contained 81.1 ± 5.8% $NKR\text{-}P1A^+$ and 1.8 ± 0.6% $CD3^+$ cells ($n = 7$), as tested in indirect immunofluorescence; $CD3^+/NKR\text{-}P1A^+$ cells were undetectable. Homogeneous NK cell populations were also purified from 10-d cultures of umbilical cord blood PBL with the B-lymphoblastoid RPMI 8866 cell line (16).

$NKR\text{-}P1A^+/CD56^+$ and $NKR\text{-}P1A^+/CD56^-$ cells were purified from day-30 cultures of Lin^- cells (see below) by indirect anti-Ig panning. For this, $CD56^+$ cells were selected (procedure repeated twice) after sensitization with anti-CD56 mAb. Homogeneous $NKR\text{-}P1A^+/CD56^-$ populations were then prepared from the $CD56^-$ cells after sensitization with B199.2. The cell populations obtained were >99% $NKR\text{-}P1A^-/CD56^-$ and $NKR\text{-}P1A^+/CD56^+$, respectively, as determined by indirect immunofluorescence.

Hematopoietic Progenitor Cell Cultures. Lin^- cells were cultured (37°C, 8% CO_2 atmosphere) in 24-well tissue culture plates (2×10^5 cells/well) in 2 ml RPMI 1640 medium (BioWhittaker Inc., Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co.) on a confluent monolayer of 30-Gy irradiated murine Sl/Sl^4 bone marrow stromal cell line or its derivative Sl/Sl^4hSCF^{220}, expressing membrane-bound SCF (24) (provided by Dr. D. Williams, University of Indiana School of Medicine, Indianapolis, IN). Recombinant (r) IL-2 (50 U/ml, sp act 1.1×10^6 U/mg protein, obtained from the Biological Response Modifiers Program, National Cancer Institute, Bethesda, MD) was added at the beginning of the cultures and every 4 d during a 30-d culture period. The culture medium was partially replaced every 7 d, and nonadherent cells from individual

wells were subcultured when confluent (once or twice, on average). These culture conditions are referred to as "primary cultures."

For secondary cultures, 10^6 NKR-P1A$^+$/CD56$^+$ and CD56 cells, separated as described above from day-30 primary cultures, were cultured for 10 d in 2 ml medium/well of 24-well culture plates. rIL-2 (50 U/ml) and rIL-12 (1 ng/ml, sp act 4.5×10^6 U/mg protein in an IFN-γ induction assay; kindly provided by Dr. S. Wolf, Genetics Institute, Andover, MA) were added at the beginning of the culture and every 4 d afterward. Daudi cells (30-Gy irradiated) were added (2×10^5 cells/well).

Immunofluorescence (Flow Cytometry). One-, two-, and three-color immunofluorescence analysis was performed with the indicated FITC-, PE-, or biotin-labeled mAb as previously described (2, 16). FITC- (Vector Laboratories, Burlingame, CA), PE- (Becton Dickinson and Co.), or R670- (Gibco BRL, Gaithersburg, MD) labeled streptavidin were used to detect biotin-labeled mAb. Samples were analyzed on an EPICS Elite, or a Profile-II flow cytometer (Coulter Corp.).

Cell-mediated Cytotoxicity. This was tested in 4-h ^{51}Cr release assays using K562 or P815 cells sensitized with anti-P815 rabbit serum as targets for spontaneous and antibody-dependent cell-mediated cytotoxicity (ADCC), respectively (2). Target cells were 10^4/well. When indicated, effector cells were incubated (5×10^6 cells/ml, 18 h, 37°C) with rIL-2 (50 U/ml) and rIL-12 (4 ng/ml) before testing.

Reverse Transcription (RT) PCR. Cells were incubated (5×10^6 cells/ml, 2 h, 37°C) or not, as indicated, in medium without or with PMA (10^{-9} M; Sigma Chemical Co.) and Ca^{2+} ionophore (A23187, 0.1 μg/ml; Sigma Chemical Co.). Total RNA was extracted from 2.5×10^5 cells using RNAzol (Biotecx Laboratories, Houston, TX) after the manufacturer's protocol. mRNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase and oligo-dT 3′ primer (37°C, 1 h). The primer pairs were designed to span intronic regions, to prevent genomic DNA amplification. CD16 cDNA was amplified (30 cycles) using previously described oligonucleotide primers and conditions (25). Primers (synthesized at the Kimmel Cancer Center Nucleic Acid Facility) were: No. 616: sense GGG GGC TTG TTG GGA GTA AA; No. 485: antisense GAG AGG CCT GAG GAT GAT. The β actin primers were derived from the murine sequence (sense TGG GAA TGG GTC AGA AGG ACT; antisense TTT CAC GGT TGG CCT TAG GGT T) (30 amplification cycles: melting, 94°C; annealing, 55°C; extension, 72°C; 1 min each). IFN-γ (sense GCA TCG TTT TGG GTT CTC TTG GCT GTT ACT GC; antisense CTC CTT TTT CGC TTC CCT GTT TTA GCT GCT GG), and TNF-α (sense GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A; antisense GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC) primers were purchased from Clontech Laboratories, Inc. (Palo Alto, CA) (30 cycles: melting, 1 min, 94°C; annealing, 1 min, 60°C; extension, 2 min, 72°C). Monocyte-specific esterase (MSE) cDNA was amplified using previously described oligonucleotide primers (sense GGC AGT TAC TCT CAG AGG CTA; antisense-CTT CCA CAG GAG TGA CAT GGC) and conditions (26). PCR products were electrophoresed in 2% agarose gels and, when indicated, transferred to nylon membrane (Hybond-N; Amersham Corp., Arlington Heights, IL) and hybridized to specific oligonucleotide probes (CD16, No. 465: TCA TTT GTC TTG AGG GTC [25]; β actin, AGC AAG AGA GGT ATC CTG ACC CTG AAG TAC CCC ATT GAA; MSE, GGT TCT TGG CCA ATG GAG ACA). These were labeled with γ-[^{32}P]ATP

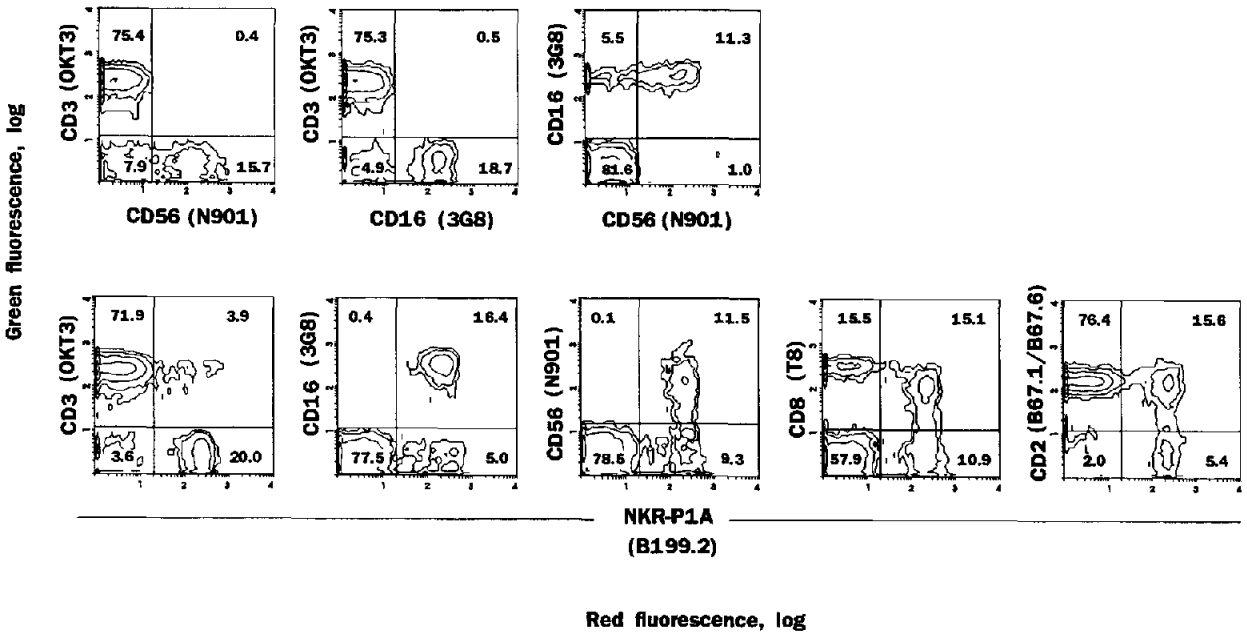

Figure 1. Surface phenotype of umbilical cord blood lymphocytes. Two-color immunofluorescence (flow cytometry) was performed with the indicated mAb on umbilical cord blood PBL. The mAb were labeled with FITC (green fluorescence), PE (red fluorescence), or biotin (detected with streptavidin-PE). Correlate measurements of red (*x* axis, log scale), and green (*y* axis, log scale) fluorescence are displayed as two-dimensional contour plots. Based on the negative control samples in the presence of FITC- and PE-streptavidin only (not shown), the contours were divided into quadrants: (*top left*) cells with green fluorescence (binding FITC-labeled antibody only); (*top right*) double-positive cells; (*bottom right*) cells with red fluorescence (binding PE-labeled antibodies only), (*bottom left*) double negative cells. Percent positive cells (background subtracted) in each quadrant are indicated. The numbers on the *x* and *y* axes are arbitrary units of fluorescence intensity (log scale).

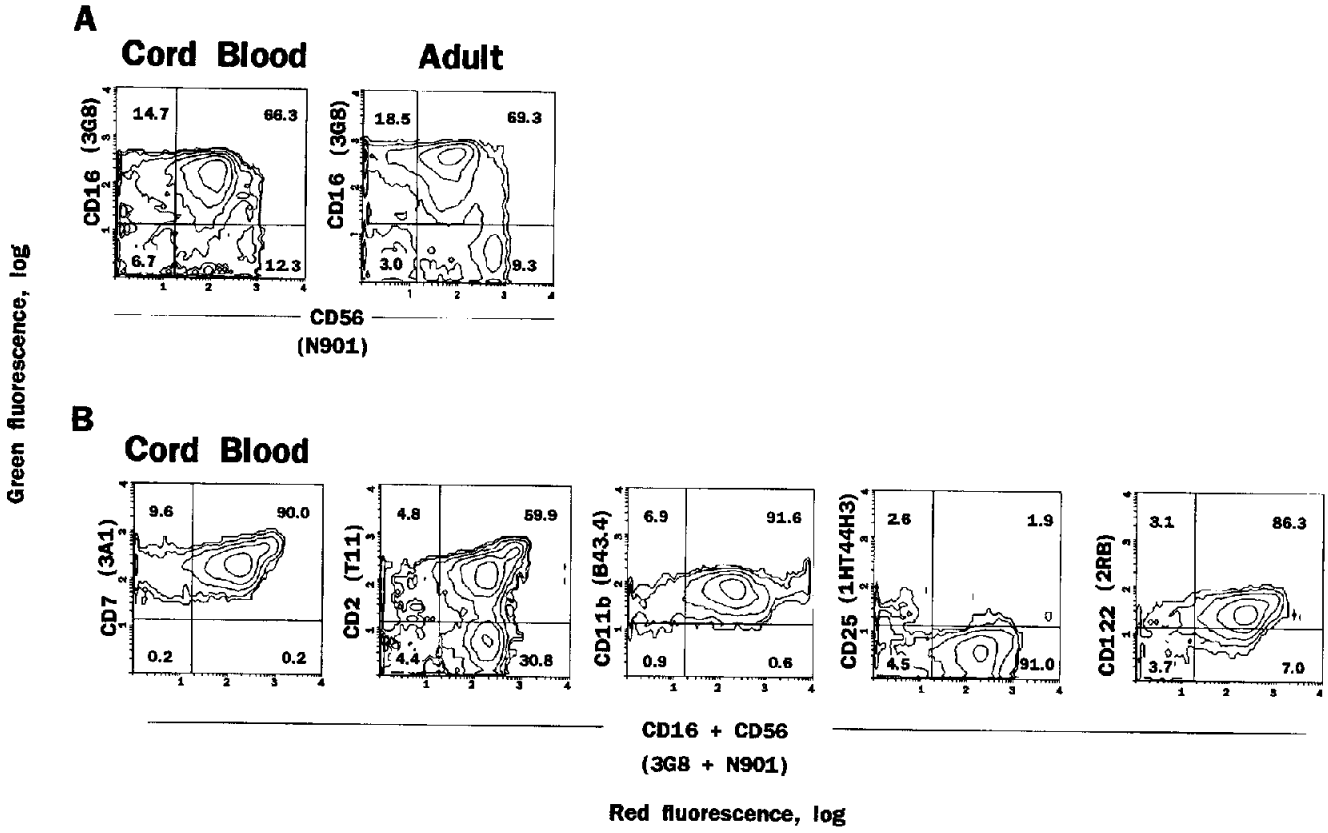

Figure 2. Identification of CD3$^-$/NKR-P1A$^+$/CD56$^-$/CD16$^-$ lymphocytes in adult and umbilical cord blood. Three-color immunofluorescence was performed on CD3$^-$ PBL. Antigen expression was analyzed on adult (*A*) and umbilical cord blood (*A* and *B*) PBL, electronically gated on NKR-P1A$^-$ cells identified with biotin-labeled B199.2 and R670-streptavidin. The indicated surface antigens were detected with FITC- or PE-labeled Ab, as described in Fig. 1. *x* axis, red, *y* axis, green fluorescence.

(sp act>3,000 Ci/mmol; NEN DuPont, Boston, MA) and T4 polynucleotide kinase (27). All enzymes and the ϕX174 DNA/HaeIII molecular weight markers were from Promega Corp. (Madison, WI).

IFN-γ and TNF-α RIA. These were performed as previously described (28, 29) on the cell-free supernatants from cells cultured for the times specified in the indicated conditions, using the mAb pairs B133.1, B133.5 (anti-IFN-γ) and B154.2, B154.7 (anti-TNF-α). Purified natural IFN-γ (Interferon Sciences, New Brunswick, NJ), and rTNF-α (provided by Dr. H.M. Shepard, Genentech Inc., South San Francisco, CA) were used as standards.

Immunocytochemistry. Cells (3×10^4) were cytocentrifuged on poly-L-lysine–treated slides, fixed in acetone (−20°C, 10 min), and stored at −80°C until use. mAb to TIA-1 (30) (IgG, 5 μg/ml; Coulter Corp.) and to IL-12 p40 (31) (C8.1, ascites, 10^{-3} dilution; provided by Dr. G. Trinchieri, Wistar Institute, Philadelphia, PA), rabbit anti–mouse perforin antiserum cross-reacting with human perforin (32) (provided by Dr. C.-C. Liu, The Rockefeller University, New York) or control nonimmune rabbit serum (both 10^{-2} dilution) were added to the rehydrated cells. After incubation and washings, positive cells were detected with biotin-labeled GaMIg or goat anti–rabbit Ig and biotin-labeled horseradish peroxidase-avidin complex (ABC kit; Vector Laboratories, Burlingame, CA) and diaminobenzidine (Sigma Chemical Co.) substrate, following the manufacturer's instructions. Staining was intensified using the diaminobenzidine intensification reagent (Vector Laboratories), and hematoxylin was used to counterstain the nuclei.

Single-cell Nα-benzyloxycarbonyl-L-lysine-thiobenzyl ester, hydrochloride esterase assays to detect serine esterase (SE)-1 were performed, according to published procedures (33), on cytocentrifuge preparations of NKR-P1A$^+$/CD56$^+$ or CD56$^-$ cells prepared as described above from 30-d cultures.

Statistical Analysis. Data were analyzed using the two-tailed, two-sample *t* test (Minitab statistical analysis software; State College, PA). $P < 0.05$ were considered significant.

Results

Identification of a CD3$^-$/NKR-P1A$^+$/CD56$^-$/CD16$^-$ Lymphocyte Population within Adult and Umbilical Cord Blood. As in PBL from adults (34, and data not shown), CD3$^-$/CD56$^+$ and CD3$^-$/CD16$^+$ NK cell subsets were detected in umbilical cord blood PBL. The majority (>70%) of the CD16$^+$ cells expressed CD56, and >90% of the CD56$^+$ cells expressed CD16, as determined by two-color immunofluorescence (flow cytometry) (Fig. 1, *top*). NKR-P1A was expressed on all CD16$^+$ and CD56$^+$ NK cells, therefore identifying most, if not all, neonatal NK cells, and on ≈5% of the CD3$^+$ T lymphocytes (Fig. 1, *bottom*). As in adult PBL (not shown) NKR-P1A was expressed on most

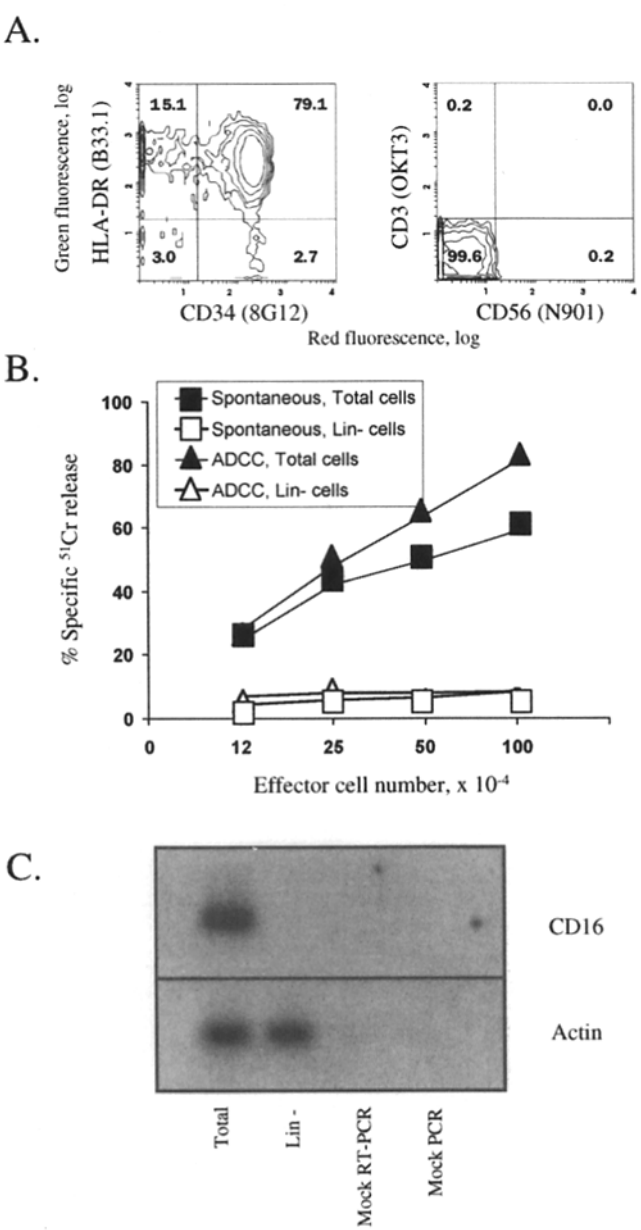

Figure 3. Phenotype of Lin cells (*A*) Two-color immunofluorescence analysis was performed on Lin^- umbilical cord blood cells as in Fig. 1; *x* axis, PE-labeled Ab; *y* axis FITC-labeled Ab. Percent positive cells per quadrant are indicated. (*B*) Spontaneous cytotoxicity (□, ■) and ADCC (△, ▲) were measured in a 4-h ^{51}Cr release assay using the indicated number of effector cells and 10^4 K562 or Ab-coated P815 target cells, respectively. Effectors were total (■, ▲) and Lin^- cells (□, △) preincubated (18 h, 37°C) with rIL-2 (50 U/ml) and rIL-12 (4 ng/ml), as described in Materials and Methods. (*C*) Expression of CD16 and β actin mRNA was analyzed by RT-PCR. Total mRNA was extracted from the indicated cell types and RT-PCR was performed as described in Materials and Methods. PCR products were electrophoresed on 2% agarose gels, transferred to nylon membranes, and hybridized to ^{32}P-labeled gene-specific internal oligonucleotide probes.

$CD8^{+dim}$ NK cells but only on a proportion of $CD8^{+high}$ T cells and $CD2^+$ cells.

Three-color immunofluorescence was performed to analyze directly the phenotype of the $CD3^-/NKR\text{-}P1A^+$ NK cells. Fig. 2 shows two-color histograms of $CD3^-$ lymphocytes purified from umbilical cord blood and electronically gated on the $NKR\text{-}P1A^+$ cells. These contained $CD16^+/CD56^+$ (68.0 ± 4.7%), $CD16^+/CD56^-$ (18.2 ± 4.3%), and $CD16^-/CD56^+$ (12.4 ± 5.9%) subsets (mean ± SD, $n = 4$). An additional $NKR\text{-}P1A^+/CD16^-/CD56^-$ subset was also detected in PBL from both neonates (4.2 ± 1.7%, $n = 4$) and an adult donor (Fig. 2 *A*). CD7 and CD11b were expressed on most umbilical cord blood $NKR\text{-}P1A^+/CD16^-/CD56^-$ NK cells while CD2 was expressed on ~50% of the cells (4.8 vs 4.4%), similar to their expression in the total NK cell population (Fig. 2 *B*). CD122 (IL-2Rβγ) and CD25 (IL-2Rα) were detected on ~50% (3.1% vs 3.7%) and 35% (2.6% vs 4.5%) of the cells in the same subset, respectively.

Generation of Noncytotoxic $NKR\text{-}P1A^+/CD56^-/CD16^-$ NK Cells from Lin^- Mononuclear Cells. The Lin^- cell populations, mostly $HLA\text{-}DR^+$, included ≈80% $CD34^+$ cells and did not contain detectable $CD56^+$ (Fig. 3 *A*), $CD16^+$, or $NKR\text{-}P1A^+$ cells (not shown), as tested by indirect immunofluorescence. They did not mediate spontaneous cytotoxicity or ADCC even after 18-h incubation with IL-2 and IL-12 (Fig. 3 *B*) and did not contain cells expressing CD16 mRNA, as detected using RT-PCR (Fig. 3 *C*) with sensitivity to detect 1 in 5×10^5 $CD16^+$ cells (not shown).

Several culture conditions (Table 1) were analyzed for their ability to support NK cells' generation from the Lin^- populations and/or mature NK cells survival/proliferation. PBL from umbilical cord blood, and $CD3^-/NKR\text{-}P1A^+$ NK cells purified from them, did not proliferate when cultured with Sl/Sl^4hSCF^{220} and IL-2, and the latter died within 14 d. Instead, significant proliferation of Lin^- cells occurred during the first 15 d of culture in the same conditions (total number of cells per well was $1.2 \pm 0.8 \times 10^6$, mean ± SD of 5–15-d cultures, $n = 7$). At this time, myelo-monocytic $CD64^+$ cells constituted the majority of the cells and a significant number of $CD34^+$ cells was present only until this time (data not shown). After a decrease at intermediate times (Fig. 4), the total cell numbers by day 30 were similar to those in the initial 2 wk ($3.3 \pm 2.1 \times 10^6$ cells per well, mean ± SD of 26–39-d cultures, $n = 4$). $NKR\text{-}P1A^+/CD3^-$ cells, expressing or not CD56 and cytotoxic to K562 target cells (not shown) were present in the cultures by day 11 ($2.3 \pm 2.9 \times 10^5$ cells per well, mean ± SD of 5–15-d cultures, $n = 7$, and representative experiment in Fig. 4). Starting at nearly day 20, their numbers increased until day 39, the last time point examined, as shown in the same figure (the total numbers of $CD3^-/NKR\text{-}P1A^+$ cells/well were $2.7 \pm 0.9 \times 10^5$, $n = 5$, and $2.1 \pm 1.0 \times 10^6$, $n = 4$, in 16–25- and 26–39-d cultures, mean ± SD).

After 20 d, two $CD3^-/NKR\text{-}P1A^+$ NK cell populations were detected in the cultures: one (40.2 ± 21.5%, mean ± SD, $n = 9$) was $CD56^+$, the other (57.9 ± 23.2%) was $CD56^-$ (Fig. 5, *top*). Most, if not all, $CD3^-$ cells expressing CD16, CD2, or CD8 were included in the $CD56^+$ subset (Fig. 5, *bottom*). CD7 and cytoplasmic CD3ε were detected in most cells in both subsets (data not shown).

Cytotoxic Activity of $NKR\text{-}P1A^+/CD56^-$ NK Cells. The

Table 1. *Generation of CD3⁻/NKR-P1A⁺ Cells from Umbilical Cord Blood Lin⁻ Cells*

Cell population*	Culture conditions	IL-2‡	CD3⁻/NKR-P1A⁺	cells/well, × 10⁴§
PBL	Sl/Sl⁴hSCF²²⁰	+	0.3, 0.3	(2)
Purified NK cells	Sl/Sl⁴hSCF²²⁰	+	nd‖	(2)
Lin⁻ cells	Sl/Sl⁴	−	nd	(2)
	Sl/Sl⁴	+	nd	(2)
	Sl/Sl⁴hSCF²²⁰	−	nd	(4)
	Sl/Sl⁴hSCF²²⁰	+	22.6 ± 15.0	(10)

*The indicated cell types were cultured for 10–25 d (2 × 10⁵ cells/well) in the conditions listed
‡rIL-2, 50 U/ml, was added every 4 d.
§The number of CD3⁻/NKR-P1A⁺ cells recovered at the end of the culture was calculated as the product of the percent positive cells (detected by immunofluorescence) by the number of total viable cells. The mean ± SD is indicated, when appropriate, and the number of experiments performed in each condition is indicated in parentheses.
‖nd, not detectable

NKR-P1A⁺ cells obtained after 25-d culture were cytotoxic to the K562 target cells (Fig. 6 *A*). The CD56⁺, but not the CD56⁻, subset purified from them was cytotoxic against the same target cells (Fig. 6 *A*). The granule-associated protein TIA-1 was expressed in 90.0 ± 3.0% of the CD56⁺ cells (mean ± SD, $n = 3$), as tested by immunocytochemistry (Fig. 6 *B*); the same protein was detected in 64.0 ± 15.0% of the CD56⁻ cells. Cells expressing SE-1 and perforin were detected in each subset in proportions similar to those seen for TIA-1 (data not shown).

Induction of NKR-P1A⁺/CD56⁻ NK Cells' Differentiation. To test the hypothesis that the NKR-P1A⁺/CD56⁻ cells were immature NK cells with potential to differentiate further, homogeneous populations of these cells were purified from day-30 cultures, and were cultured for 10 additional days in the conditions listed in Table 2. Cell viability was maintained in all conditions, but a significant proportion of CD56⁺ cells (43.2 ± 25.9%, mean ± SD, $n = 7$) was detected only in cultures with irradiated Daudi cells, IL-2, and IL-12 ($P < 0.05$) and the cells from these cultures, like those from cultures of the CD56⁺ subset (not shown) were cytotoxic to K562 target cells (Fig. 7). NKR-P1A expression and viability of the CD56⁺ subset were maintained throughout secondary culture in each of the culture conditions (data not shown).

As shown in Fig. 8, NKR-P1A⁺/CD56⁻ cells from 30-d cultures expressed no detectable IFN-γ mRNA even after stimulation with phorbol diester and Ca^{2+} ionophore. Instead, IFN-γ mRNA was detected in the same populations after 10-d secondary culture. Both CD56⁺ (not shown) and CD56⁻/NKR-P1A⁺ cells (Fig. 8) expressed TNF-α mRNA constitutively both before and after secondary culture. Minimal amounts of IFN-γ and TNF-α were present in the supernatants from primary cultures (up to day 30); instead, both cytokines were detected at significant levels in cell-free supernatants collected during secondary culture (Table 3).

Discussion

In this study, we identify a discrete subset of immature NKR-P1A⁺/CD56⁻ human NK cells and its requirements for differentiation to phenotypically mature CD56⁺ NK cells capable of cytotoxicity and IFN-γ production.

NK cells were generated in cultures containing SCF and IL-2. Because mature NK cells could not be generated in the absence of SCF, both cytokines are necessary for this process. Shibuya et al. (9) proposed that, in the presence of SCF, CD34⁺ early NK cell progenitors in the bone marrow are sequentially sensitive to IL-3 and IL-2, and become sensitive to IL-2 and SCF independent only after they reach the stage of differentiation at which they express CD122 and CD56. Our data support a minimal require-

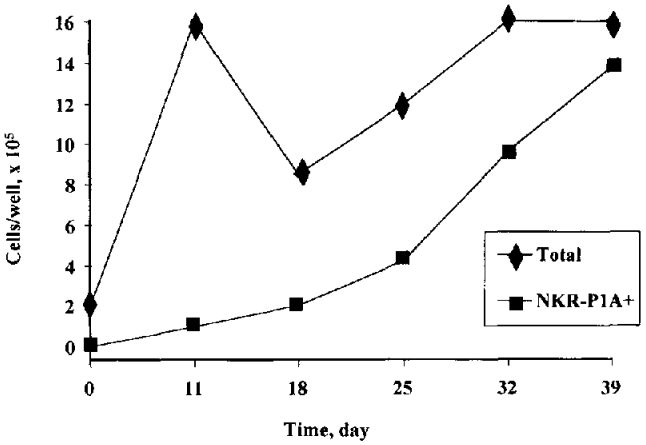

Figure 4. Kinetics of generation of NKR-P1A⁺ cells. Lin⁻ cells (2 × 10⁵/well) were cultured on Sl/Sl⁴hSCF²²⁰ cells with IL-2. The percentage of CD3⁻/NKR-P1A⁺ cells was evaluated by immunofluorescence on the indicated days, and the number of viable cells was counted. The absolute number of cells expressing NKR-P1A was calculated as the product of the percentage of mAb⁺ cells by the number of viable cells present at the indicated time points. ◆, total cells; □, NKR-P1A⁻ cells (representative experiment).

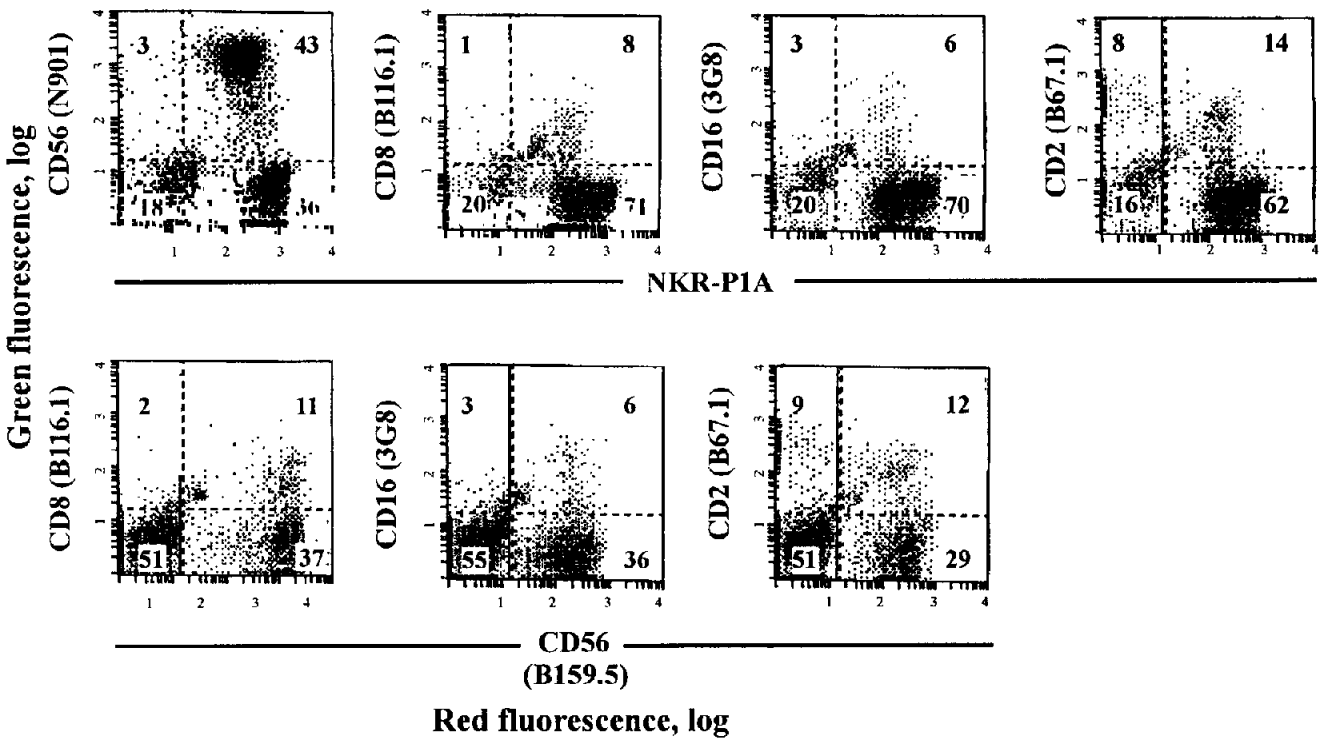

Figure 5. Surface phenotype of day-28 primary culture cells. Lin^- cells were cultured as in Fig. 4. Two-color immunofluorescence analysis was performed, as in Fig. 1, using the indicated FITC- and PE-labeled antibodies, on cells collected after 28-d cultures. *x* axis, red fluorescence (PE); *y* axis, green fluorescence (FITC).

ment for SCF and IL-2 in NK cell differentiation in vitro, but do not exclude a role for other cytokines including, but possibly not limited to, IL-3 and produced by cells in the heterogeneous $CD34^+$ or Lin^- populations. One such cytokine is likely represented by IL-15 (11). Cytokines possibly produced by the stromal cell line used as feeder are unlikely to be relevant to this process, because most murine cytokines are ineffective on human cells. These findings contrast with reports indicating that IL-2 is sufficient for NK cell maturation (6). However, in that study formal proof of the absence of contaminating mature NK cells was not presented. Mature NK cells proliferate in response to IL-2 (35) and SCF-induced proliferation of a minor subset of c-*kit* $receptor^+$ NK cells has been reported (36). We exclude that IL-2–dependent proliferation of residual mature NK cells in the Lin^- cell populations accounts for the NK cells generated in our culture conditions because: (*a*) IL-2 alone did not support NK cell generation from the Lin^- populations; (*b*) Lin^- cells do not mediate spontaneous cytotoxicity or ADCC; (*c*) $CD56^+$ and/or NKR-$P1A^+$ cells, or cells expressing CD16 mRNA are not detectable in the Lin^- cells by immunofluorescence or RT-PCR, respectively; (*d*) mature NK cells do not proliferate in conditions that support, instead, NK cell differentiation from the Lin^- cells; and (*e*) the surface phenotype and functional properties of the cells generated in culture differ from those of most circulating NK cells, including the $CD56^{+bright}$/c-*kit* $receptor^+$ NK cell subset reported by Matos et al. (36). These data also indicate that the proliferation/maturation requirements of mature and immature NK cells are distinct.

Unlike previous reports, we detected two distinct NKR-$P1A^+$ NK cell populations in the cultures. Similar to NK cells derived in other culture conditions (6, 8, 10, 12), one subset is phenotypically and functionally mature and expresses CD56 and, although only on a minor proportion of the cells, other surface antigens of mature NK cells, e.g., CD16, CD2, and CD8. It is possible that CD16 represents a late NK cell differentiation antigen, as is the case for myeloid cells (37), that the culture conditions provided do not allow complete phenotypic maturation, and/or that cytokines inducing CD16 expression, e.g., platelet-derived growth factor (38), are missing.

The second subset, NKR-$P1A^+/CD56^-$, is functionally and phenotypically immature and has a surface phenotype similar, if not identical, to that of the NKR-$P1A^+/CD56^-/CD16^-$ subset we have identified in both adult and neonatal PBL. The existence of circulating immature NK cells had been postulated by Nagler et al. (34), who described a $CD56^+/CD16^-$ PBL subset that, unlike mature NK cells, expresses IL-2Rα, once stimulated with IL-2 proliferates in response to IL-4, and does not express IFN-γ mRNA. Being $CD56^-$ and not cytotoxic, the $CD16^-/$NKR-$P1A^+$ cells described here are distinct from that population. Given the limited number of circulating NKR-$P1A^+/CD16^-/CD56^-$ cells, no functional data are available yet for these cells isolated directly from PBL; however,

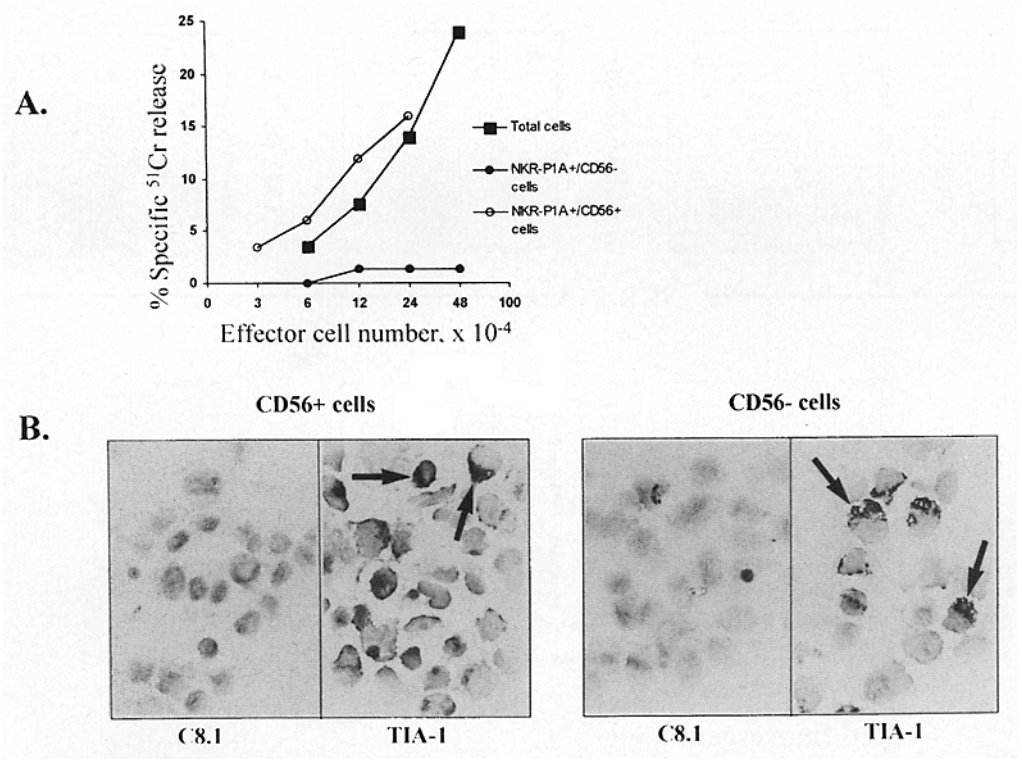

Figure 6. Cytotoxic activity and TIA-1 expression in NKR-P1A$^+$ cell subsets NKR-P1A$^+$/CD56$^+$ or CD56$^-$ cells were separated from 30-d cultures of Lin$^-$ cells, as described in Fig 4 and in Material and Methods. (*A*) Spontaneous cytotoxicity of total (■), CD56$^+$ (○), and CD56$^-$ (●) NKR-P1A$^+$ cells was measured in a 4-h ^{51}Cr-release assay with the indicated number of effector cells and 10^4 K562 target cells/well. Experiment representative of five performed. (*B*) Cytoplasmic expression of TIA-1 was analyzed by immunohistochemistry. mAb C8.1 (anti-IL-12, p40) was used as negative control. Arrows indicate cytoplasmic staining. Magnification, 500.

based on the in vitro data, it can be hypothesized that these cells represent circulating immature NK cells that can be recruited to functional maturation.

Several possibilities may explain the presence in our cultures of both mature NK cells and those in an apparently arrested state of development. Lin$^-$ populations are heterogeneous and may contain NK cell progenitors at different stages of differentiation and with distinct growth factor requirements. The most mature ones, as previously proposed (9, 12), may respond readily to IL-2, and/or be induced to differentiate by factors endogenously produced or added exogenously. More immature progenitors may need additional factors that are not provided or produced in the cultures. Alternatively, and/or in addition, the culture conditions likely change during the course of 30 d; e.g., myelo-monocytic cells, which may produce factors affecting NK cell differentiation (39) are present only at early times, and any cell only partially differentiated by the time these changes occur may be unable to mature completely.

The distinctive marker of the immature NK cell subset reported here is NKR-P1A. This is a type II integral membrane protein with a region of sequence homology with C-type lectins in its extracellular domain (21) and 47% homology with rodent NKR-P1, shown to transduce activatory signals (40) and capable of binding specific carbohydrate moieties (41, 42). The role and the natural ligand(s) of this molecule, and its biological significance on human NK and T cells is still to be defined, but its usefulness as a marker of NK cell development is clear. NKR-P1A expression on NK cells differentiated from immature progenitor cells has not been analyzed in previous reports, and it remains to be established whether the subset identified here can be generated from purified CD34$^+$ cells, as those used in most other reports. NK cell progenitors are enriched in the CD7$^+$/CD34$^+$ cell population (7). Unlike CD7, NKR-P1A is absent on umbilical cord blood CD34$^+$ cells, but becomes expressed on NK cells within 7 d in the primary cultures described (data not shown), indicating that the NKR-P1A$^+$/CD56$^-$/CD7$^+$ cells represent NK cells at a later stage of differentiation than the CD34$^+$/CD56$^-$/CD7$^+$ ones.

The NKR-P1A$^+$/CD56$^-$ cells are not cytotoxic. The possibility that their functional incompetence is only apparent, and limited to the target cells used, cannot be excluded. However, the observation that after secondary culture these cells can kill the same targets suggests that this is not the case. The inability of these cells to kill is unlikely to depend, primarily, on lack of cytotoxic granule-associated proteins because TIA-1 and perforin were present in a significant percentage of the cells, and SE-1 was functionally active both in total cell lysates and at the single cell level (not shown). Also, surface adhesion molecules known to be involved in target cell recognition (the LFA-1/CD18 complex) (43, 44) are expressed on the NKRP1A$^+$/CD56$^-$ cells at a density similar to that on the CD56$^-$ cells (data not shown). These data support the hypothesis that lack of cytotoxicity depends on the absence of one or more functional cytotoxicity-triggering elements in the CD56$^-$ cells: these may include target recognition receptor(s) yet to be identified and/or biochemical pathways of signal transduction. Irrespective of the reason, our data indicate that expression of cytotoxic granule-associated proteins precedes functional maturation of the machinery needed for target cell recognition and triggering of cytotoxicity. This con-

Table 2. *Induction of CD56 Expression on NKR-P1A$^+$/CD56$^-$ Cells*

Culture conditions*	Total cell number/well, × 10^6; mean ± SD (n)‡	Percent CD56$^+$ cells; mean ± SD (n)‡
IL-2	0.9 ± 0.6 (3)	6.3 ± 6.9 (3)
IL-2 + IL-12	0.8 ± 0.6 (3)	9.5 ± 5.6 (3)
Daudi + IL-2	0.8 ± 0.1 (3)	8.0 ± 6.9 (3)
Daudi + IL-2 + IL-12	0.9 ± 0.2 (7)	43.2 ± 25.9 (7)§

*NKR-P1A$^+$/CD56$^-$ cells (10^6 cells/well) were purified from 30-d primary cultures and cultured for 10 d in the conditions listed, as described in Materials and Methods. rIL-2, 50 U/ml; rIL-12, 1 ng/ml; 30-Gy irradiated Daudi cells, 2 × 10^5/well.
‡Percent CD56$^+$ cells was determined by immunofluorescence; n, number of experiments performed.
§$P < 0.05$, Daudi + IL-2 + IL-12 vs each of the other conditions; two-tailed, two-sample t test.

clusion is also supported by the reported existence of a noncytotoxic immature NK1.1, NKR-P1C$^+$ (45), NK cell subset in mice with ablated bone marrow (15). Like the human NKR-P1A$^+$ cells described here (data not shown), these cells bind targets, lack other differentiation markers characteristic of mature NK cells, and have been hypothesized to represent NK cells that are frozen at an immature stage of differentiation and that, to undergo complete maturation, require factors provided only in the environment of an intact bone marrow. Similarly, our culture conditions likely lack factors, present in vivo, needed for complete NK cell differentiation.

The NKR-P1A$^+$/CD56$^-$ subset is induced to develop into functional NK cells in conditions different from those supporting the initial differentiation of Lin$^-$ cells along the NK cell lineage. IL-2, either alone or in combination with feeder cells, is insufficient to induce further phenotypic maturation of these cells and only the addition of IL-12 allows this process, indicating that IL-12 is necessary for the maturation of NK cell progenitors in vitro. Given the limited number of cells available for these studies, we tested cytotoxicity only in the cultures in which phenotypically mature cells could be detected, and we cannot exclude the possibility that the cells derived from the cultures in the presence of IL-2, alone or with the B lymphoblastoid cell line, although phenotypically immature, are also cytotoxic.

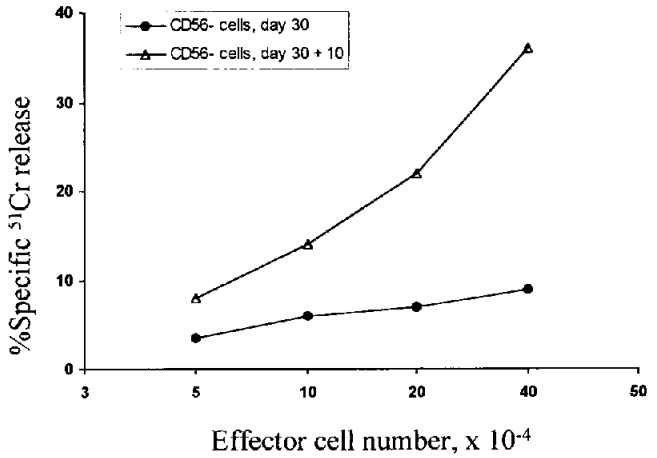

Figure 7. Cytotoxic activity of NKR-P1A$^+$/CD56$^-$ cells before and after secondary culture. CD56$^-$ cells were purified from 30-d primary culture (●) and cultured for 10 d in secondary cultures (△) as described in Materials and Methods. Spontaneous cytotoxicity was measured in a 4-h ^{51}Cr release assay using K562 target cells. Experiment representative of three performed.

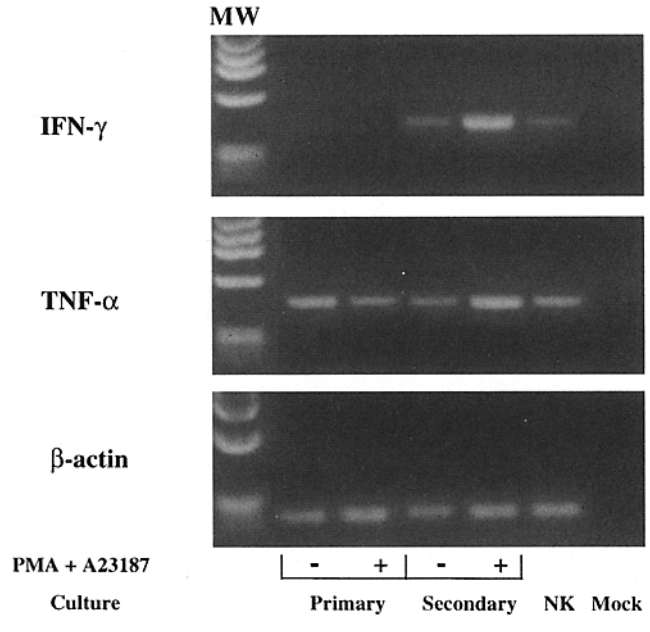

Figure 8. IFN-γ and TNF-α mRNA expression in NKR-P1A$^+$/CD56$^-$ cells before and after secondary culture. Cells were prepared and cultured as in Fig. 7. RT-PCR analysis was performed on RNA extracted from the cells after 30-d primary cultures and after secondary culture with IL-2, IL-12, and Daudi cells, as indicated. After separation, cells were incubated without (−) or with (+) PMA (10^{-9} M) and Ca^{2+} ionophore (A23187, 0.1 μg/ml) for 2 h. The RT-PCR products were visualized after ethidium bromide staining, after agarose gel electrophoresis. Mol wt markers were ϕX174 DNA/HaeIII. *NK*, umbilical cord blood NK cells; *Mock*, no mRNA/cDNA template in either RT or PCR.

Table 3. *Cytokine Production in Primary and Secondary Cultures*

Culture conditions*	Cell population	IFN-γ, U/ml ± SD (*n*)‡	TNF-α, U/ml ± SD (*n*)‡
Primary culture, day 15	Total cells	1.3 ± 1.6 (3)	1.6 ± 1.7 (3)
Secondary culture, day 8	$CD56^+$	486.0 ± 198.0 (3)	161.0 (1)
	$CD56^-$	532.0 ± 101.0 (3)	319.0 (1)

*In primary cultures, Lin^- cells were cultured (2×10^5 cells/well) on Sl^4hSCF^{220} feeder cells with rIL-2 (50 U/ml). The CD3 /NKR-P1A$^+$ cell subsets, purified from 30-d cultures, were incubated, separately, with 30-Gy irradiated Daudi cells to which rIL-2 (50 U/ml) and rIL-12 (1 ng/ml) were added on days 0, 4, and 7

‡IFN-γ and TNF-α were detected by RIA in the cell-free supernatants collected on the days indicated. *n*, number of experiments performed.

Based on the data in the murine system (45), however, we consider this unlikely. Whether the B lymphoblastoid feeder cells are necessary (possibly providing cell–cell contact or cytokines), or IL-12 is sufficient in the absence of IL-2 and/or feeder cells, and whether the effect is mediated by this cytokine directly, or indirectly via induced production of additional factors, is under investigation. In IL-12 p40 knockout mice (46) the absolute number and subset composition of NK cells have not been analyzed; however, only a modest, though significant, decrease in spontaneous cytotoxicity has been reported. On this basis we consider it likely that, even if proven sufficient to support late NK cell differentiation, IL-12 is not the sole cytokine able to mediate this effect. One likely candidate for this is IL-15, that induces NK cell differentiation from $CD34^+$ cells (11) and, in the culture conditions reported here, can induce generation, from Lin^- cells, of NK cells that are phenotypically more differentiated than those induced by IL-2 (Zamai, L., E. Rosati, I.M. Bennett, and B. Perussia, manuscript in preparation). IL-12 induces myeloid cell differentiation acting with SCF, IL-3, or GM-CSF (47, 48). Unlike IL-2 and IL-15 (11), IL-12 does not support NK cell differentiation when used instead of IL-2 under identical primary culture conditions (48): thus, the effects of IL-12 on NK cell differentiation appear to be stage-specific. Whether or not IL-15 has similar effect on the NKR-P1A$^+$/$CD56^-$ immature NK cells is under investigation.

Mature NK cells produce several cytokines including IFN-γ and TNF-α (49, and reviewed in reference 50). The NKR-P1A$^+$/$CD56^-$ cells, like the $CD56^+$/$CD16^-$ cell subset described by Nagler et al. (34), express TNF-α but not IFN-γ mRNA after primary culture. TNF-α expression is unlikely to reflect myeloid cells contamination because MSE mRNA was not detectable in the same conditions, and cells with myeloid phenotype were absent. The presence of TNF-α mRNA in both the $CD56^+$ and the $CD56^-$ NKR-P1A$^+$ cell subsets suggests that expression of this cytokine occurs early during NK cell maturation, whereas IFN-γ production represents a more specialized function, acquired only at later differentiation stages. The possible biological significance of this remains to be determined.

Based on these observations, it may be speculated that at the onset of an immune response to intracellular pathogens IL-12 plays a role to activate maximally the nonadaptive system of defense, in which NK cell are potent and essential effectors, both enhancing the functional activity of mature NK cells, and inducing maturation of circulating precytotoxic NK cells.

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